บทความวิจัย

การกำหนดขอบเขตของชนิดโดยใช้หลายโลไซและการประมาณอัตราการเกิดชนิดใหม่ภายใน *Cladia aggregata* complex (Lecanorales, Ascomycota)

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บทคัดย่อ

ความสัมพันธ์เชิงวิวัฒนาการของไลเคน *Cladia aggregata* complex ศึกษาโดยใช้ข้อมูลลำดับนิวคลีโอไทด์บริเวณ ribosomal nuclear ITS และ IGS, protein-coding *Mcm7* และ protein-coding *GAPDH* โดยวิธี maximum likelihood และ Bayesian approach ร่วมกับเกณฑ์ที่ใช้กำหนดขอบเขตของชนิด ได้แก่ Genealogical Concordance of Phylogenetic Species Recognition ผลการศึกษาโดยอาศัยความสอดคล้องของข้อมูลลำดับนิวคลีโอไทด์จากทั้ง 4 โลไซ สามารถกำหนดขอบเขตของชนิดได้อย่างน้อย 12 สายวิวัฒนาการ และแสดงการเพิ่มขึ้นของอัตราการเกิดชนิดใหม่อย่างเด่นชัดภายใน *Cladia aggregata* complex โดยในแต่ละชนิดที่มีความเป็นไปได้ส่วนใหญ่ขาดลักษณะทางสัณฐานวิทยาที่เด่นเฉพาะ จึงบ่งบอกถึงความหลากหลายที่ยังคงซ่อนเร้นอยู่ภายใน *Cladia aggregata* complex นอกจากนี้ ลักษณะทางสัณฐานวิทยาบางอย่าง อาทิเช่น รูปแบบการแตกแขนง สีของแทลลัส แอสโคสปอร์ และพิกนิดิโอสปอร์แสดงลักษณะร่วมที่พัฒนาแล้วแต่ไม่ได้มีจุดกำเนิดเดียวกัน อย่างไรก็ตาม การใช้ข้อมูลฟีโนไทป์แบบรวมสามารถใช้แยกความแตกต่างในบางสายวิวัฒนาการได้

คำสำคัญ: *Cladia aggregata,* GCPSR, อัตราการเกิดชนิดใหม่

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Multi-Locus Species Delimitation and Estimating Speciation Rate within the *Cladia aggregata* complex (Lecanorales, Ascomycota)

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ABSTRACT

Phylogenetic studies of the *Cladia aggregata* complex were carried out using the ribosomal nuclear ITS and IGS, protein-coding *Mcm7* and protein-coding *GAPDH* DNA sequences. The maximum likelihood and Bayesian approach methods together with species recognition criterion namely, Genealogical Concordance of Phylogenetic Species Recognition were employed. The result based on concordance of genealogies obtained from 4 nuclear loci recognized 12 lineages and apparently increased diversification rate within the *C. aggregata* complex. Most of the putative species lacked phenotypically diagnostic characteristics, indicating that there is a high level of cryptic diversity in the *Cladia aggregata* complex. Moreover, some morphological characteristics such as branching pattern, thallus surface colors, ascospores and type of pycnidiospores appeared to have homoplastic synapomorphies. However, a few clades can be characterized by a combination of several phenotypic characters.

Keywords: *Cladia aggregata,* GCPSR, Diversification rate

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Introduction

Lichens are symbiotic organisms composed of microscopic green algae or cyanobacteria and filamentous fungi. They take the external shape of the fungal partner and hence are named based on the fungus. The largest number of lichenized-fungi occurs in the Ascomycota and rarely a member of the Basidiomycota. Lichens have three major types, including foliose, crustose and fruticose lichens [1]. Some lichens survive in the tolerance conditions of deserts, and others on frozen soil of the arctic regions.

Traditionally, species circumscriptions in lichen-forming fungi are based on phenotypic characters, such as thallus and ascomatal morphology and anatomy, or chemical characters, such as the presence of secondary metabolites. Environmental factors have been shown to influence phenotypic characters in various groups of lichens [2-4]. Also a remarkable amount of morphological disparity within clades has been demonstrated in several clades of lichenized fungi [5-11] calling the use of these characters to delimit taxa in question. Furthermore, it has repeatedly been shown that the traditional species delimitation underestimates the diversity of these fungi with numerous cryptic lineages discovered under currently accepted species in various unrelated families [12-15]. The group studied here is the *Cladia aggregata* complex which is a difficult group of lichenized fungi. Previous classifications have accepted between one [16] and eight species [3] based on different interpretations of morphological and chemical diversity in the group. This genus is especially diverse in Tasmania with a number of chemo-and morphotypes only known from this island [3]. The genus *Cladia* belongs to Cladoniaceae (Lecanorales, Ascomycota) which currently includes 16 genera with over 400 accepted species [17]. Most genera in this family have a dimorphic thallus with a crustose or foliose primary thallus and a vertical secondary thallus that bears fruiting bodies [18-19]. A few genera in the family also have foliose thalli. *Cladia* spp. have a crustose primary thallus and a fruticose, secondary thallus, often referred to as pseudopodetium [20].

To address the species diversity on the basis of concordance of multiple gene genealogies in the *C. aggregata* complex, a data set using four loci, including internal transcribed spacer of nuclear ribosomal DNA (nuITS rDNA), 28S-18S ribosomal DNA intergenic spacer (IGS), the protein-coding nuclear glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* gene and the DNA replication licensing factor *(Mcm7)* gene were generated. The molecular data were used to perform phylogenetic reconstructions in a maximum likelihood (ML) and Bayesian (B/MCMC) framework. Moreover, to understand the processes leading to diversification in this group of lichens, a lineage-through-time (LTT) plot was examined.

Materials and Methods

Taxon sampling

The taxon sampling included most morpho-and chemotypes known from the *Cladia aggregata* group and two samples of *C. schizopora* as outgroup based on previous molecular studies [5, 6]. Data of 126 representative samples were assembled using nuITS, IGS, the protein-coding *GAPDH* and *Mcm7* genes. Specimens from several countries, including Australia, Brazil, Colombia, Cuba, India, La Réunion, New Zealand, Peru and Thailand were studied. These specimens encompassed the *Cladia aggregata* complex with seven recognized chemotypes. The chemical constituents were identified using thin layer chromatography (TLC) [21, 22] and gradient-elution high performance liquid chromatography (HPLC) [23].

Molecular methods

Total DNA was extracted from herbarium material using the DNeasy Plant Mini Kit (Qiagen) following the instructions of the manufacturer. Dilutions (10^{-1} up to 10^{-2}) of DNA were used for PCR amplifications. Primers for PCR amplifications, PCR and cycle sequencing conditions were as described previously [5, 6]. Sequence alignments were carried out separately for each data set using BioEdit [24]. Ambiguous regions in the nuITS and IGS alignments were removed manually before analysis.

Evolutionary model selection

The best fit evolutionary model of all partitions was performed using the program jModelTest v.0.1.1 [25, 26]. Taxa missing particular loci were excluded for the model selection. The best model for each loci retrieved from the test of 24 models of substitution was implemented under Bayesian approach and maximum likelihood.

Phylogenetic analyses

To test for potential conflict, parsimony bootstrap analyses were performed on each individual data set, and 70% bootstrap consensus trees were examined for conflict [27]. Since no conflicts (i.e. well supported differences in the topology) were found, multi-gene data sets were analyzed under maximum likelihood Bayesian approach.

A Bayesian analysis was performed using MrBayes 3.1.2 [28] with the GTR+G model for the protein-coding genes *(Mcm7 & GAPDH),* the GTR+I+G model for IGS and SYM+G model for nuITS rDNA. The data sets were partitioned into four parts, including IGS, nuITS, the protein-coding *GAPDH* and *Mcm7* genes. Each partition was allowed to have its own

parameter values. No molecular clock was assumed. Heating of chains was set to 0.2. Posterior probabilities were approximated by sampling trees using a variant of Markov Chain Monte Carlo (MCMC) method. Number of generations was 10 million. To avoid autocorrelation, only every 1000^{th} tree was sampled. The first 4,000 generations were discarded as burn in. We plotted the log-likelihood scores of sample points against generation time using TRACER v1.4.1 (http://tree.bio.ed.ac.uk/software/tracer/) to ensure that stationarity was achieved after the first 4,000 generation by checking whether the log-likelihood values of the sample points reached a stable equilibrium value [28]. Additionally, we used AWTY [29] to compare split frequencies in the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. Of the remaining 19992 trees (9996 from each of the parallel runs) a majority rule consensus tree with average branch lengths was calculated using the sumt option of MrBayes. Posterior probabilities were obtained for each clade. Only clades with posterior probabilities ≥ 0.95 in the Bayesian analysis were considered as strongly supported. Phylogenetic trees were visualized using the program Treeview [30].

The ML analysis of the concatenated alignment was performed with the program RAxML-HPC2 (version 7.3.1) on XSEDE [31] using the default rapid hill-climbing algorithm. The model of nucleotide substitution chosen was GTRGAMMA. The data set was partitioned into eight parts (IGS, nuITS and each codon position of *GAPDH* and *Mcm7* genes), so each gene partition was treated as an independent data set. Rapid bootstrap estimates were carried out for 1000 pseudoreplicates.

Diversification analyses

For the diversification analyses, the data set was pruned to include only one sample per species (with the exception of the *Cladia aggregata* samples that did not form a monophyletic group). To obtain ultrametric trees, the maximum likelihood tree was used as a starting tree in an MCMC tree sampling procedure under Bayesian evolutionary analysis by sampling trees: Beast v. 1.4.5 [32] using the lognormal relaxed clock model [33] with the same model as in the Bayesian phylogenetic analyses. The tempo of lineage accumulation (speciation minus extinction) was visualized by plotting the natural logarithm of the number of lineages against the branch length distance from the root node of the chronogram. The lineage-through-time plot was calculated for the entire *Cladia aggregata* clade using Mesquite version 2.75 with the Diverse package [34-36].

Results

Phylogenetic analyses

A data matrix of 2219 unambiguously aligned characters, with 520 characters in nuITS, 396 characters in IGS, 793 characters in the *GAPDH* gene and 510 characters in the *Mcm7* gene, was used for phylogenetic analyses. The data set included 1610 constant characters. The general time-reversible model with a gamma distribution and/or invariant model of rate heterogeneity was employed for analyses of the single-locus and concatenated data sets. Since no strongly supported conflicts between the four single-locus ML phylogenetic trees were detected, a combined data set was analyzed. In the B/MCMC analysis of the combined data set, the likelihood parameters in the sample had the following averaged values for the nuITS, IGS, *GAPDH* and *Mcm7* partitions (± standard deviation): base frequencies $\pi(A) = 0.2102$ (± 0.0004), $\pi(C) = 0.2648$, $\pi(G) = 0.2575$ (± 0.0004), $\pi(T) = 0.2675$; rate matrix $r(AC) = 5.7339$ (± 0.1462), $r(AG) = 0.3068$ (± 0.0003), $r(AT) = 6.0973$ (± 0.1580), $r(CG) = 3.6219$ (± 0.1295), $r(CT) = 0.4789$ (± 0.0014), $r(GT) = 5.9799$ (± 0.1117) and the gamma shape parameter

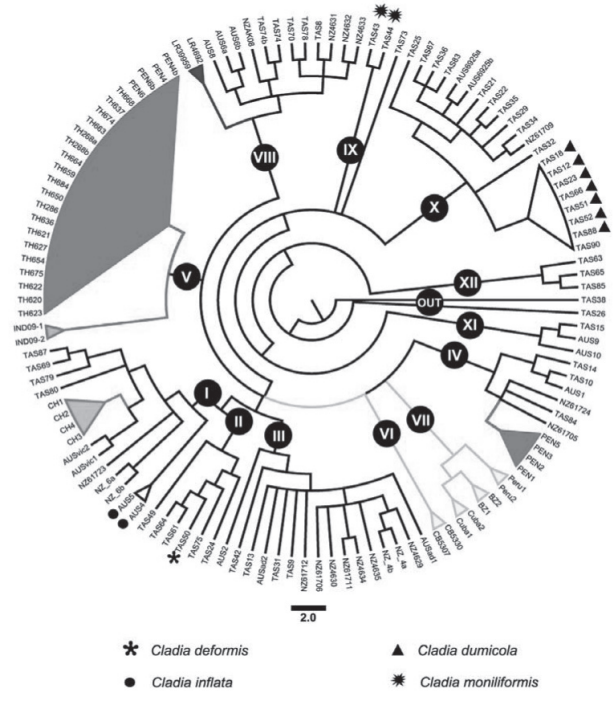

Figure 1 Phylogenetic tree reconstructed following B/MCMC & ML methods. The tree was depicting relationships of the *Cladia aggregata* complex based on a combined analysis of nuITS, IGS, the protein-coding *GAPDH* and *Mcm7* genes. The scale bar indicates the number of nucleotide substitutions per site.

$\alpha = 0.5819$ (± 0.0004). The likelihood parameters in the sample had a mean likelihood of LnL = -11728.85 (± 0.354), while the ML tree had a likelihood of LnL = -9763.28.

The specimens included the seven chemotypes in the *Cladia aggregata* complex and four of the currently described species in addition to *C. aggregata, viz. C. deformis, C. dumicola, C. inflata,* and *C. moniliformis.* The phylogenetic estimate of the ML and B/MCMC analyses were congruent; hence the Bayesian trees are shown here (Figures 1 and 2). The *Cladia aggregatas.* lat. samples fall into 12 main, well-supported clades (Figures 1 and 2). Some clades consist of strongly supported subclades. There are some associations between clades and chemotypes as well as geographical origins. Of the 12 clades obtained, five have restricted

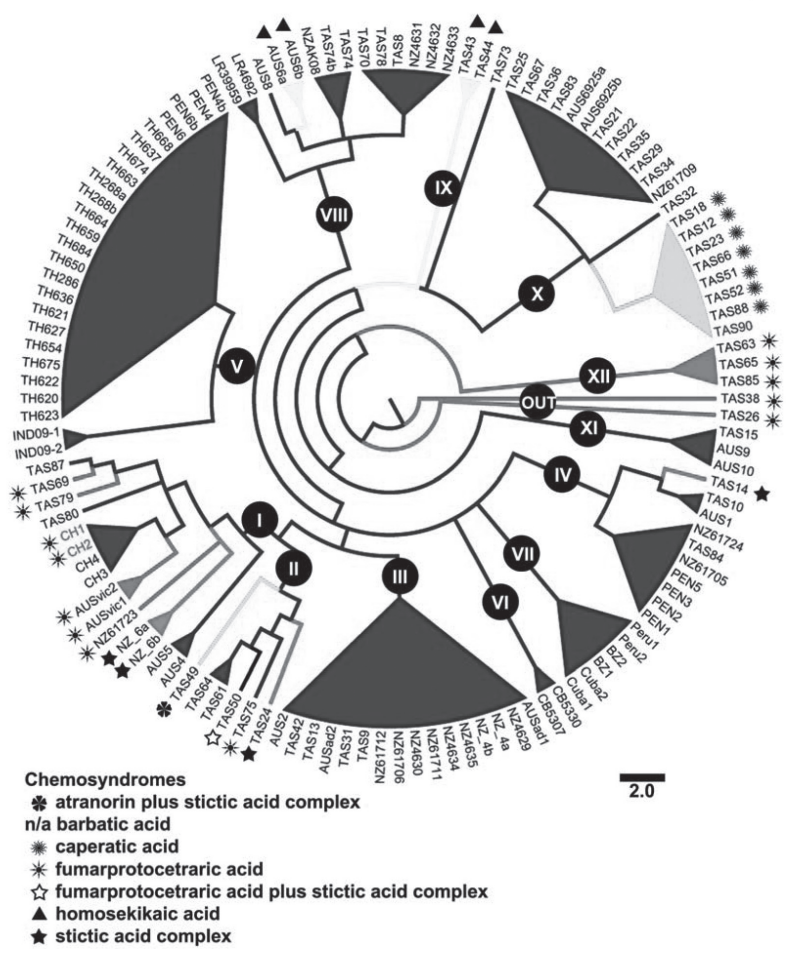

Figure 2 Phylogenetic tree reconstructed following B/MCMC & ML methods. The tree was depicting relationships of the *Cladia aggregata* complex chemosyndromes based on a combined analysis of nuITS, IGS, the protein-coding *GAPDH* and *Mcm7* genes. The scale bar indicates the number of nucleotide substitutions per site.

distributional ranges (clades II, VI-VII, IX and XII). Clades II, IX and XII are restricted to Tasmania (TAS), whereas clades VI and VII occur in the Neotropics, including Brazil (BZ), Colombia (CB), Cuba and Peru. Seven clades correspond with the chemotypes (clades III, V-VII, IX, XI and XII). Clades III, V-VII and XI contain only members of the barbatic acid chemosyndrome. Clade IX includes the homosekikaic acid chemosyndrome. Clade XII reveals the fumarprotocetraric acid chemosyndrome.

Clade I (BS=99 & PP=1.00) includes samples from two currently accepted species, viz. *C. aggregata* and *C. inflata.* This clade has an austral distribution, occurring in Australia, New Zealand and southern South America (southern part of Chile (CH)). Two samples of *C. inflata* are clustering together and form a sister group within this clade. Three chemotypes are found in this clade, including the barbatic, fumarprotocetraric and stictic acid chemosyndromes. **Clade II** (BS=98 & PP=1.0) contains a chemically diverse group of samples all collected in Tasmania. It includes samples of *C. aggregata* and *C. deformis.* Five chemotypes present are (1) atranorin plus stictic acid complex, (2) barbatic acid, (3) fumarprotocetraric acid, (4) fumarprotocetraric acid plus stictic acid complex and (5) stictic acid complex. **Clade III** (BS=99 & PP=1.0) includes *C. aggregata* samples from Australasia (including New Zealand (NZ)) which possess the barbatic acid chemosyndrome. **Clade IV** (BS=79 & PP=1.00) includes *C. aggregata* from Australasia and Southeast Asia (Penang (PEN)). This clade comprises species containing the stictic acid and barbatic acid chemosyndromes. **Clade V** (BS=100 & PP=1.0) consists entirely of members currently placed in *C. aggregata* containing the barbatic acid chemosyndrome and occurring in Southeast Asia (Thailand (TH) and Penang) and India (IND). All Neotropical collections of *C. aggregata* are clustered together in **Clades VI** (BS=100 & PP=1.00) and **VII** (BS=100 & PP=1.00) and contain the barbatic acid chemosyndrome. **Clade VIII** (BS=92 & PP=1.00) includes *C. aggregata* samples from Australasia and La Réunion (LR) island (east of Madagascar) with two types of chemosyndromes: the barbatic acid and homosekikaic acid chemosyndromes. Two morphologically distinct samples of *C. moniliformis* form **Clade IX** (BS=100 & PP=1.00) and contain the homosekikaic acid chemosyndrome. So far, *C. moniliformis* is only known from Tasmania. **Clade X** (BS=100 & PP=1.00) comprises specimens from Australasia containing either the barbatic acid or caperatic acid chemosyndrome. While the majority of barbatic acid containing samples in this clade forms one subclade, the other subclade includes only two barbatic acid containing samples which are intermixed with the caperatic acid containing samples of *C. dumicola.* **Clade XI** (BS=100 & PP=1.00) includes *C. aggregata* with barbatic acid chemosyndrome from Australia. **Clade XII** (BS=100 & PP=1.00) consists of *C. aggregata* specimens collected in Tasmania. All samples in this clade contain the fumarprotocetraric acid chemosyndrome.

Diversification analyses

The combined data set of the four loci was used to produce a chronogram. The log number of lineages was plotted against the relative time of each node. A straight line with slope equaled to the per lineage speciation rate was expected. The lineage-through-time plot showed a cumulative number of lineages closer to the tips of the chronogram (Figure 3).

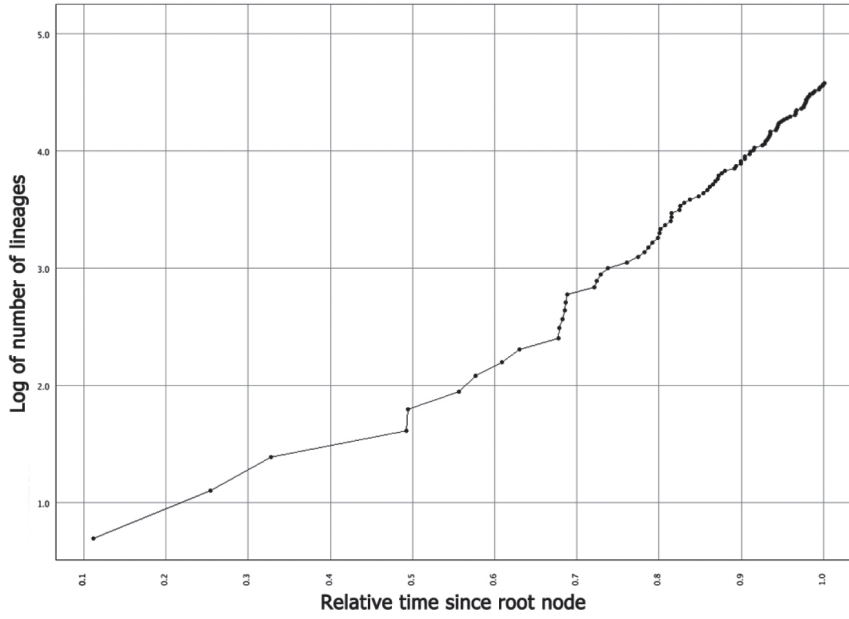

Figure 3 Lineage-through-time plot for the entire *Cladia aggregata* clade.

Discussion and Conclusions

On the basis of Genealogical Concordance Phylogenetic Species Recognition (GCPSR) criterion the strong supported branches should separate genetically isolated species due to coalescence of alleles, while poorly supported branches should be found within a recombining species [12, 37]. Hence, 12 putative species were recognized based on this criterion (Figure 1).

This study is another example for the presence of cryptic species in lichen-forming fungi challenging the phenotypically-based species circumscription in these organisms [38, 39]. This is especially true for species complexes in which morphological and chemical diversity has previously been documented [33, 34]. The *Cladia aggregata* complex is an extremely controversial group with no obvious correlation of morphological and chemical variation. Some morphological and/or chemical variants have previously been recognized as distinct species. These include *Cladia inflata* with inflated pseudopodetia [39] and *C. moniliformis* with

inflated pseudopodetia plus bulbous segments, while others were predominantly recognized based on their chemistries [3]. The previously described species *Cladia deformis, C. inflata* and *C. dumicola* were not supported in their current circumscriptions by our analyses. In fact, all previously recognized species in the *C. aggregata* group, except *C. moniliformis* (Figure 1), did not form distinct clades. The barbatic acid chemosyndrome occurred in almost all clades and/or subclades (Figure 2). Hence, the presence of chemosyndromes alone does not indicate the affiliation of a sample to a lineage in these fungi. Individuals within each of the chemotypes were morphologically variable (*i.e.* clade III: *C. aggregata* with the barbatic acid chemosyndrome) and individuals in different chemotypes often shared each of morphological traits (*i.e.* clade II: *C. aggregata* and *C. deformis*) (Figures 1 and 2). This could be due to the fact that chemical and some morphological characteristics, such as branching pattern, surface colors, ascospores and type of conidia are homoplasious. The absence of a correlation of secondary chemistry and phylogenetic placement may be due to different reasons. It seems obvious that they share ancestral polymorphisms and might retain some features of their ancestor's morphology. Moreover, the lineage-through-time plot suggested that the increase in speciation rate towards the present was found in the *C. aggregata* complex. This phenomenon can be explained by an actual increase in the diversification rate or an increase caused by a constant background extinction rate [40].

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